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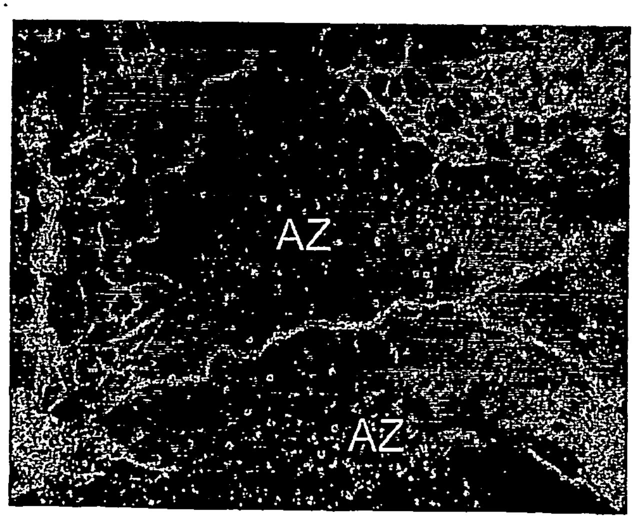
(54) Title: COMPOUNDS AND METHODS FOR PROMOTING ANGIOGENESIS BY USING A GAMMA-SECRETASE INHIBITOR OR INHIBITING THE GAMMA-SECRETASE PATHWAY



(57) Abstract: Angiogenesis may be initiated or increased through the use of gamma-secretase inhibitors. The gamma-secretase inhibitor DAPT can initiate and increase angiogenesis. Methods for initiating and increasing angiogenesis are used for disease prevention and treatment as well as for generating research models.

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Title of the Invention

Compounds and methods for promoting angiogenesis by using a gamma-secretase inhibitor or inhibiting the gamma-secretase pathway.

Field of the Invention

The invention relates to the field of angiogenesis and treatment of diseases or conditions related to angiogenic abnormalities.

Background of the Invention

Angiogenesis is a fundamental process required for the normal growth and development of tissues, and involves the proliferation of new capillaries from preexisting blood vessels. Under normal physiological conditions, humans or animals only undergo angiogenesis in very specific situations and angiogenesis is tightly controlled through a highly regulated system of angiogenic stimulators and inhibitors. Deviation from such a tight control often leads to or is associated with disease.

Angiogenesis is a prerequisite for the development and differentiation of the vascular tree, as well as for a wide variety of fundamental physiological processes including embryogenesis, somatic growth, tissue and organ repair and regeneration, cyclical growth of the corpus luteum and endometrium, and development and differentiation of the nervous system. In the female reproductive system, angiogenesis occurs in the follicle during its development, in the corpus luteum following ovulation and in the placenta to establish and maintain pregnancy. Angiogenesis additionally occurs as part of the body's repair processes, e.g. in the healing of wounds and fractures. Angiogenesis is also a factor in tumor growth, since a tumor must continuously stimulate growth of new capillary blood vessels in order to grow.

Both controlled and uncontrolled angiogenesis are thought to proceed in a similar manner. Endothelial cells and pericytes, surrounded by a basement membrane, form capillary blood vessels. Angiogenesis begins with the erosion of the basement membrane by enzymes released by endothelial cells and leukocytes. The endothelial cells, which line the lumen of blood vessels, then protrude through the basement membrane. Angiogenic stimulants induce the endothelial cells to migrate through the eroded basement membrane. The migrating cells form a "sprout" off the parent blood vessel, where the endothelial cells undergo mitosis and proliferate. The endothelial sprouts merge with each other to form capillary loops, creating the new blood vessel. *See* e.g. Folkman *et al.,* Adv. Cancer Res., Vol. 43, pp. 175-203 (1985), and Ingber *et al.,* Cell, Vol. 58, pp. 803-805 (1985).

While persistent, unregulated angiogenesis occurs in numerous disease states, insufficient or nonexistent angiogenesis can also be a serious medical problem. Promoting angiogenesis is desirable in situations where vascularization is to be established or extended, for example after tissue or organ transplantation, or to stimulate establishment of collateral circulation in tissue infarction or arterial stenosis, such as in coronary heart disease and thromboangitis obliterans. Enhancing angiogenic activity may also be useful in treating ischemic conditions, including cardiovascular and limb ischemia. Finally, materials or methods that initiate or increase angiogenesis

could potentially also be used to create research models with greater-than-normal angiogenesis.

The protease gamma-secretase is a complex of at least four proteins: presenilin 1 (PS 1), nicastrin, APH-1, and PEN-2. Gamma-secretase has more than one enzymatic activity cleaving multiple substrates. It is also involved in processing the Notch receptor, part of a signalling pathway critical for embryonic development. The importance of this pathway is seen in knockout PS-1 mice which die *in utero* or shortly after birth (Shen *et al.*, 1997, *Skeletal and CNS defects in Presenilin-1-deficient mice,* Cell; 89(4):629-39), understood to be at least partially due to PS-1's role in normal or sufficient angiogenesis (Nakajima M, *et al.*, June 2003, *Abnormal blood vessel development in mice lacking presinilin-1,* Mech Dev 120(6):657-67). Researchers have thus found it desirable to both further define gamma-secretase itself, as well as to identify compounds which interact with gamma-secretase, such as inhibitors. (N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-Butyl Ester or DAPT is a cell-permeable dipeptide protease inhibitor, one of the known gamma-secretase inhibitors which blocks Notch signaling (Micchelli, C.A. *et al.*, 2003, *gamma-secretase/presenilin inhibitors for Alzheimer's disease phenocopy Notch mutations in Drosophila,* FASEB J. 17, 79-81).

In addition to its' interest as a research tool, numerous diseases can be linked to gamma-secretase or the gamma-secretase pathway. For example, gamma-secretase is involved in Alzheimer's Disease ("Alzheimer's"). Alzheimer's is characterized by the formation of plaques. The plaques are mainly comprised of beta-amyloid peptides (A beta). In addition to their role in Alzheimer's, A beta are produced during normal cellular metabolism. The peptides are 40-42 amino acids in length and are derived from larger amyloid precursor proteins. To date, this family of proteins is understood to contain three members: amyloid precursor protein (APP), amyloid precursor-like protein 1 (APLP 1), and amyloid precursor-like protein 2 (APLP2).

At least two pathways are involved in the processing of these proteins, which can result in, *inter alia,* the formation of A beta. Under normal conditions, APP may be cleaved extracellularly by alpha-secretase to create a membrane-bound intermediate. This intermediate is subsequently cleaved by gamma-secretase to release a non-amyloidogenic fragment. In an alternate pathway, smaller quantities of APP are cleaved extracellularly by beta-secretase (BACE1) to form a membrane-bound intermediate. This intermediate is subsequently cleaved by gamma-secretase to release the amyloidogenic A beta fragment (Scheinfeld *et al.,* 2002, J. Biol. Chem. 277:44195-201).

Because of their obvious utility in the treatment and prevention of Alzheimer's alone, numerous compounds such as DAPT which block gamma-secretase through a variety of means have been identified and developed. But while significant research has been done identifying gamma-secretase and inhibitors thereof, as well as diseases and abnormal conditions related to gamma-secretase, there remains a need in the art to

completely identify the mechanisms of action of gamma-secretase and utilize this knowledge to improve modern medicine.

Summary of the Invention

It is therefore an object of the present invention to provide materials or methods that initiate or increase angiogenesis. It is further an objection of the present invention to describe new uses for gamma-secretase inhibitors. According to the invention, gamma-secretase inhibitors have surprisingly been shown to increase angiogenesis. This novel elucidation of activity is exploited in treatments for angiogenesis and related conditions.

According to a first embodiment of the invention, an angiogenesis initiator or increaser is provided which comprises a pharmaceutically-effective amount of a gamma-secretase inhibitor. The gamma-secretase inhibitor may be DAPT. The angiogenesis initiator or increaser may further include a pharmaceutically acceptable carrier or adjuvant.

According to a further embodiment of the invention, a method of influencing a disease state in a cell, a group of cells, or an organism is provided, which comprises administering at least one of a gamma-secretase inhibitor or a gamma-secretase pathway inhibitor to the cell, group of cells, or organism. The disease thus influenced can be selected from the group consisting of atherosclerosis, hemangioma, hemangioendothelioma, vascular malformations, warts, pyogenic granulomas, hair growth, Kaposi's sarcoma, scar keloids, allergic edema, neoplasms, psoriasis, decubitus or stasis ulcers, gastrointestinal ulcers, dysfunctional uterine bleeding, follicular cysts, ovarian hyperstimulation, endometriosis, neoplasms, preeclampsia, placental insufficiency, respiratory distress, ascites, peritoneal sclerosis, adhesion formation, metastatic spreading, coronary artery disease, ischemic heart disease, ischemic limb disease, obesity, rheumatoid arthritis, synovitis, bone destruction, cartilage destruction, osteomyelitis, pannus growth, osterphyte formation, cancer, aseptic necrosis, impaired fracture healing, hepatitis, pneumonia, glomerulonephritis, asthma, nasal polyps, liver regeneration, pulmonary hypertension, systemic hypertension, diabetes, retinopathy of prematurity, diabetic retinopathy, choroidal disorders, intraocular disorders (e.g. age related macular degeneration), leukomafacia, stroke, vascular dementia, disease, thyroiditis, thyroid enlargement, thyroid pseudocyst, tumor metastasis, lymphoproliferative disorders, lympgoedema, AIDS, and hematologic malignancies. The gamma-secretase inhibitor could be DAPT or an analogue of DAPT.

According to a further embodiment of the invention, a method of increasing the angiogenic process in a cell, a group of cells, or an organism is provided which comprises administering a pharmaceutical composition comprising a pharmaceutically effective amount of at least one gamma-secretase inhibitor or gamma-secretase pathway inhibitor to the cell, group of cells, or organism. The gamma-secretase inhibitor could be DAPT or an analogue of DAPT. The pharmaceutical composition

may be administered to prevent, treat, or cure a condition selected from any of the above-noted disease states.

According to a further embodiment of the invention, a method for initiating or increasing angiogenesis in a cell, a group of cells, a tissue, or an organism is provided, which comprises inhibiting the gamma-secretase pathway in the cell, group of cells, tissue or organism. A further method is provided which initiates or increases angiogenesis in a cell, a group of cells, a tissue, or an organism by inhibiting gamma-secretase in the cell, group of cells, tissue or organism. The methods may comprise reducing the level of expression of gamma-secretase, administering DAPT, administering an antibody against gamma-secretase, or delivering a vector to the target, wherein the vector comprises a polynucleotide encoding at least one gamma-secretase inhibitor, operatively linked to a suitable promoter. The promoter may be a tissue or organ specific promoter specific for a tissue or organ in which angiogenesis is to be initiated or increased.

According to a further embodiment of the invention, a method for screening for a substance which initiates or increases angiogenesis is provided which comprises measuring an activity of the gamma-secretase pathway in the presence of a candidate compound, measuring an activity of the gamma-secretase pathway in the absence of a candidate compound, and then comparing the activity measured in the presence of a candidate compound with the activity measured in the absence of the candidate compound. A change in activity indicates that that candidate initiates or increases angiogenesis.

As used herein "gamma-secretase inhibitor" means any material or compound that, e.g., binds to, partially or totally blocks activity, decreases, prevents, delays activation, inactivates, desensitizes, or down regulates the activity or expression of gamma-secretase or the gamma-secretase pathway. Inhibitors include genetically modified versions of gamma-secretase proteins, e.g., versions with altered activity, as well as naturally occurring and synthetic ligands, antagonists, agonists, antibodies, small chemical molecules and the like. Inhibitor, as the term is used herein, includes but is not limited to an antagonist.

The present invention encompasses compounds and compositions which have are pharmaceuticals or have a pharmaceutical effect. The compounds of the invention may be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds for assisting in uptake, distribution and/or absorption. They encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, local anesthetics, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact with the active compound.

The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a patient.

The phrase "pharmaceutically effective amount" is used herein to mean an amount sufficient to initiate or increase to some beneficial degree, preferably to increase by at least about 30 percent, more preferably by at least 40 percent, more preferably by at least 50 percent, more preferably by at least 60 percent, more preferably by at least 70 percent, more preferably by at least 80 percent, most preferably by at least 90 percent, angiogenesis as compared to untreated controls.

The compounds and compositions disclosed herein may be administered by any route, including intradermally, subcutaneously, orally, intraarterially or intravenously.

The concentration of a disclosed compound in a pharmaceutically acceptable mixture will vary depending on several factors, including the dosage of the compound to be administered, the pharmacokinetic characteristics of the compound(s) employed, and the route of administration. Skilled workers can extrapolate the mouse data presented herein, which is based on 100mg/kg, 0.1-1μM plasma concentration, to reach the desired effect in the organism of interest. The agent may be administered in a single dose or in repeat doses.

As used herein "organism" refers to animals, preferably mammals, more preferably mammals such as experimental mammals or humans. Likewise the subject to be treated by the inventive methods can mean either a human or non-human animal.

As used herein, "vector" or "expression vector" refers to a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a host cell as known in the art. The expression vector can be part of a plasmid, virus, or nucleic acid

fragment. Typically, the expression vector includes a nucleic acid to be transcribed operably linked to a promoter.

Brief Description of the Drawing Figures

Figure 1 shows the central region of a control mouse retina;
Figure 2 shows the central region of a treated mouse retina;
Figure 3 shows the capillary enclosed areas of a control mouse retina;
Figure 4 shows the capillary enclosed areas of a treated mouse retina;
Figure 5 depicts quantitative data on retinal vessel density;
Figure 6 shows labeled astrocytes in a control mouse retina;
Figure 7 shows labeled astrocytes in a treated mouse retina;
Figure 8 shows a retinal cross section with asterisks marking vascular tufts;
Figure 9 shows vascular tufts in a control mouse retina;
Figure 10 shows the absence of vascular tufts in a treated mouse retina;
Figure 11 depicts quantitative data on retinal vascular tuft formation;
Figure 12 depicts quantitative measurements of VEGF-A levels in mouse retinas;
Figure 13 shows vascularization of a control mouse retina;
Figure 14 shows vascularization of a control mouse retina;
Figure 15 shows vascularization of a treated mouse retina; and
Figure 16 shows vascularization of a treated mouse retina.

Detailed Description

The findings of the present invention are particularly useful in modulating conditions characterized by systemic or local abnormalities in angiogenic activity. Examples include, but are not limited to, eye diseases (e.g., AMD, retinopathy of prematurity, diabetic retinopathy), responses to organ transplantation, coronary artery disease, ischemic heart disease, wound healing, peripheral vascular disease, tumorogenesis/cancer, and inflammatory conditions (e.g., rheumatoid arthritis).

While the novel use of gamma-secretase inhibitors to initiate or increase angiogenesis is the most significant finding of the present invention, this knowledge can also help further advance medicine in fields where inhibition of both gamma-secretase and angiogenesis is desired.

The examples described below employed murine cells or tissues because they provided a convenient way to analyze factors upregulated in mammalian embryos. One skilled in the art will recognize that the inventive methods and compositions are also applicable to other mammals including, but not limited to, mice, rats, rabbits, dogs, pigs, and humans. For example, murine models have been extrapolated to Alzheimer's in humans. C. *elegans* and *Drosophila* species have been used to elucidate Alzheimer's related pathways with good reproducibility in mice. The close homology between mammalian genes when compared to the non-mammal models is further evidence that the data from one species of mammal is applicable to other

mammals. The same is also true with regard to the observations with regard to the gamma-secretase pathway (see e.g. Zambrowicz *et al.*, 2003, *Knockouts model the 100 best-selling drugs- will they model the next 100?,* Drug Discovery; 2:38-51; Murakami *et al.*, 2003, *Presenilin-dependent gamma-secretase activity mediates the intramembranous cleavage of CD44,* Oncogene; 22(10): 1511-6; Kopan R., *et al.*, 2004, *Gamma-secretase: proteasome of the membrane?,* Nat Rev Mol Cell Biol. 5(6):499-503.).

The present disclosure that the gamma-secretase pathway and factors active therein influence angiogenesis forms the basis for treatment methods of many human and animal diseases. The invention also encompasses kits and reagents adapted to the subject methods.

A person of ordinary skill in the art will readily recognize that a large number of potential gamma-secretase pathway inhibitors are already available (see Table 1 below). According to one embodiment, DAPT is used as an inhibitor of the gamma-secretase pathway. Additional inhibitors of gamma-secretase pathway can be identified. For example, medicinal and combinatorial chemistry methods well-known to those skilled in the art can be used to modify known PS-1 antagonists to form new gamma-secretase inhibitors with improved efficacy for the purposes of the present invention. Further, as DAPT has already been identified as one useful compound according to the invention, analogs of DAPT may be used.

Table 1: Gamma-secretase inhibitors

Gamma-Secretase Inhibitor	Comments
APP β-Secretase Inhibitor (H-KTEEISEVN-stat-VAEF-OH)	A potent inhibitor of the amyloid precursor protein (APP) β-secretase (IC_{50}-30 nM).
Balfilomycin A1, *Streptomyces griseus*	A macrolide antibiotic, acts as a specific inhibitor of vacuolar-type H^+-ATPase (V-type; *S. grieus* K_I – 500 pM). Reported to inhibit β-secretase activity and release of Aβ peptide in HEK293 cells transfected with the "Swedish" mutation APP.
OM99-2	A peptidomimetic, highly potent, tight binding transition-state analog inhibitor of β-secretase (K_I – 1.6 nM, recombinant memapsin-2; K_I - 9.58 nM, recombinant pro-memapsin 2). Designed from the template of the β-secretase site of Swedish β-amyloid precursor protein (APP) with Asp to Ala replacement. Also includes a nonhydrolyzable hydroxyrethylene isostere between Leu and Ala.
β-Secretase Inhibitor II (Z-VVL-CHO)	A potent, cell-permeable, and reversible inhibitor of β-secretase. Corresponds to the β -secretase cleavage site (VNL-DA) of the Swedish mutant amyloid precursor protein (APP). Inhibits the formation of both $A\beta_{total}$(IC_{50}–700 nM) and $A\beta_{1-42}$ (IC_{50}-2.5 pM) in CHO cells transfected with wt APP751.
γ-Secretase Inhibitor I (Z-LLnL-CHO)	Inhibits γ-secretase activity and the turnover of APP-C100.
γ-Secretase Inhibitor II	A reversible and selective peptidomimetic inhibitor of β-

	secretase (IC_{50} – 13 pM for total inhibition of Aβ). Displays only weak inhibitory activity against calpain II (IC_{50}-100pM in a purified enzyme assay).
γ-Secretase Inhibitor III (Z-LL-CHO)	A cell-permeable, reversible inhibitor of β-secretase that reduces the formation of both (IC_{50} -35pM) and $A\beta_{1-42}$ using CHO cultures stably transfected with amyloid precursor protein-751. Reported to be nontoxic and specific for γ-secretase.
γ-Secretase Inhibitor IV (2-Naphthoyl-VF-CHO)	A cell-permeable, reversible inhibitor of β-secretase. Equipotently inhibits the release of $A\beta_{1-40}$(ED_{50}-2.6 pM) and $A\beta_{x-40}$-(ED_{50}-2.7pM) in HEK293 cells stably transfected with the amyloid precursor protein Swedish mutants.
γ-Secretase Inhibitor V (Z-LF-CHO)	A cell-permeable, reversible inhibitor of β-secretase that is reported to inhibit the release of $A\beta_{x-40}$ (ED_{50}-5.0 pM) in HEK293 cells stably transfected with the amyloid precursor protein Swedish mutants.
γ-Secretase Inhibitor VI	A cell-permeable, reversible inhibitor of $A\beta_{42}$ production (IC_{50}-1.8 pM). Treatment of HEK293 cells with this inhibitor results in increase in β-secretase-cleaved APP fragments and secreted Appsβ, but no change in secreted APPsβ.
γ-Secretase Inhibitor VII (MOC-LL-CHO)	A cell-permable, reversible inhibitor of Aβ and p3 secretion ($A\beta_{40}$ IC_{50}-2.3 pM; $A\beta_{42}$ IC_{50}-3mM). Reported to be more potent (IC_{50}-900 nM and 740 nM for $A\beta_{40}$ and $A\beta_{42}$ respectively) in the presence of C99 Inhibitor; 10 pM; Cat. No. 205533).
γ-Secretase Inhibitor VII (DFK 11)	A selective, reversible inhibitor of aspartyl protease that blocks γ-secretase activity (IC_{50}-10-25 pM). Displays similar potency and selectivity for inhibition of $A\beta_{40}$ and $A\beta_{42}$ production.
γ-Secretase Inhibitor IX (DAPT)	A cell-permeable dipeptide that reduces AB production by blocking γ-secretase ($A\beta_{total}$ IC_{50}-115 nM, Aβ42 IC_{50}-200 nM). Reported to be functionally active in both HEK 293 cells and neuronal cultures without affecting the secretion of amylois-B precursor protein.
γ-Secretase Inhibitor X (L-685,458)	A cell permeable hydroxyethylene dispeptide isostere that acts as a highly specific and potent inhibitor of γ-secretase ($A\beta_{total}$ - IC_{50} – 17nM, $A\beta_{40}$ IC_{50} – 48 nM; and $A\beta_{42}$ IC_{50}-67 nM in SHSY5Y cells overexpressing spβA4CTF). Binds to PS1 and PS2 and blocks Notch intracellular domain production in neuronal cells. Functions as a transition state analog mimic at the catalytic site of an aspartyl protease. Exhibits over 100-fold greater selectivity for γ-secretase than for cathepsin D.
γ-Secretase Inhibitor XI (7-Amido-4-Chloro-3-methoxyisocoumain)	An active site-directed, irreversible serine protease inhibitor that acts as a highly selective, potent inhibitor of γ-secretase. Blocks production of both amyloid-β_{40} ($A\beta_{40}$ and $A\beta_{42}$,50% inhibition at <100 pM) in HEK293 cells expressing wild-type and Swedish mutant B-amyloid precursor protein.
γ-Secretase Inhibitor XII (Z-IL-CHO)	A cell-permeable, reversible dipeptide aldehyde that reduces AB production by blocking γ-secretase *in vitro* ($A\beta_{40}$ IC_{50} - 7.9pM; $A\beta_{42}$ IC_{50} -7.6 pM) in in cultured CHO 2b-7 cells that stably overexpress PP695 ($A\beta_{40}$ IC_{50} -11.5pM; $A\beta_{42}$ IC_{50} -8.3

	pM). Also blocks the generation of CTF-γ (γ-secretase-generated C-terminal fragment). Does not affect the formation of amyloid β precursor protein.
γ-Secretase Inhibitor XIII (Z-YIL-CHO)	A selective, reversible inhibitor of γ-secretase. In TPA-stimulated T47-14 cells, it abolishes nuclear localization of ErbB4 receptor tyrosine kinase by inhibiting the formation of the s80 ErbB4 fragment.
γ-Secretase Inhibitor XIV	A potent cell-permeable, reversible inhibitor of γ-secretase ($A\beta_{40}$ IC_{50} -190 nM; $A\beta_{42}$ IC_{50} – 780 nM) in solubilized membrane preparations and in cultured APP695 expressing CHO 2b-7 cells ($A\beta_{40}$ IC_{50} -80 nM; $A\beta_{42}$ IC_{50} -120 nM).
γ_{40}-Secretase Inhibitor I (t-3, 5, DMC-IL-CHO)	A potent cell permeable, reversible inhibitor of γ-secretase that preferentially inhibits the secretion of $A\beta_{1\text{-}40}$(>90%) vs. $A\beta_{42}$(-15%). IC_{50} - 15 pM; $A\beta_{total}$ - 22pM for $A\beta_{1\text{-}40}$; and > 50 pM for $A\beta_{42}$ in CHO cells stably transfected with the cDNA encoding βAPP695. Reported to be about 10-fold more active than Z-Val-Phe-CHO (MDL 28170; Cat. No. 208722).
γ_{40}-Secretase Inhibitor II (BOC-GW-CHO)	A cell-permeable substrate-based (β-40 site) β-secretase inhibitor that is reported to preferentially (90%) inhibit Aβ cleavage at site 40 vs. 42, in a dose-dependent fashion, in transiently transfected 293T cells overexpressing APP695NL.

In order to evaluate the efficacy of the inhibitors for the purposes of the present invention, a variety of assays are conducted to evaluate their ability to initiate or increase angiogenesis. Examples of assays are well-known to those of ordinary skill in the art, see e.g. Murray, Angiogenesis protocols, *in* Murray, Methods in Molecular Medicine, 2001, ISBN 0-89603-687-7. Known gamma-secretase inhibitors rely on the previously-elucidated understanding of the role of the gamma-secretase pathway, making some inhibitors more and some inhibitors less effective at influencing angiogenesis. Accordingly, known factors may also be evaluated for their ability to create the results desired for the novel application disclosed herein.

Assays for a gamma-secretase inhibitor that creates the desired effect on angiogenesis may also rely on its role on tumor growth. Cell lines or animal models with a known propensity for tumorogenesis can be subjected to treatment with a candidate inhibitor. Tumor growth can be monitored and evaluations can be made of vascular parameters in the tumor and vascular density and morphology in biopsies from the tumor. These results can be compared with known or control values to indicate the efficacy of the gamma-secretase inhibitor/angiogenesis increaser tested.

A skilled worker could utilize materials in the art to determine how inhibitors, thus created, could be most effectively administered. Administration may preferentially be oral. Parenteral administration could also be utilized, particularly where the properties of the gamma-secretase inhibitor and any vehicles or diluents employed are not compatible with oral uptake and distribution. Dosing of gamma-secretase inhibitors would be based on the pharmacology of the inhibitor or inhibitor mixture, including consideration of IC50 values, metabolism, excretion and toxicity values.

Administration may be for the purpose of managing disease, treating disease, preventing disease, research or other purposes.

Disease states that are related to abnormal angiogenesis and could therefore be influenced with gamma-secretase inhibitors according to the present invention are known in the art. See, for example, Carmeliet and Jain. *(Angiogenesis in cancer and other diseases,* 2000, Nature 407:249-257). Examples of diseases which may be particularly affected by compounds and methods of the present invention include atherosclerosis, hemangioma, hemangioendothelioma, vascular malformations, warts, pyogenic granulomas, hair growth, Kaposi's sarcoma, scar keloids, allergic edema, neoplasms, psoriasis, decubitus or stasis ulcers, gastrointestinal ulcers, dysfunctional uterine bleeding, follicular cysts, ovarian hyperstimulation, endometriosis, neoplasms, preeclampsia, placental insufficiency, respiratory distress, ascites, peritoneal sclerosis, adhesion formation, metastatic spreading, coronary artery disease, ischemic heart disease, ischemic limb disease, obesity, rheumatoid arthritis, synovitis, bone destruction, cartilage destruction, osteomyelitis, pannus growth, osterphyte formation, cancer, aseptic necrosis, impaired fracture healing, hepatitis, pneumonia, glomerulonephritis, asthma, nasal polyps, liver regeneration, pulmonary hypertension, systemic hypertension, diabetes, retinopathy of prematurity, diabetic retinopathy, choroidal disorders, intraocular disorders (e.g. age related macular degeneration), leukomafacia, stroke, vascular dementia, disease, thyroiditis, thyroid enlargement, thyroid pseudocyst, tumor metastasis, lymphoproliferative disorders, lympgoedema, AIDS, and hematologic malignancies.

Methods are available for monitoring the gamma-secretase pathway, which can facilitate, *inter alia,* analysis of the efficacy of inhibitors. For example, monitoring activity of the gamma-secretase pathway could be accomplished by creating a substrate for the gamma-secretase that can be detected in various assays. For example, Kinoshita *et al.* (2002, J Neurochem. 82:839-47) describes that the gamma secretase-generated carboxyl-terminal domain of APP (APP-CT) interacts in the cytoplasm with an adapter protein, Fe65, and this CT domain, when tagged with green fluorescent protein (GFP), may serve as a readout for processes that modify gamma secretase release of the APP-CT. APP-CT, when stabilized by FE65, translocates to the nucleus in a manner dependent upon stabilization by the adapter protein Fe65, and this translocation may be observed with laser scanning confocal microscopy. The APP-CT domain continues to interact with Fe65 in the nucleus, as determined by both colocalization and fluorescence resonance energy transfer (FRET). Alternatively, BRET2 (Bioluminescence Resonance Energy Transfer), as available commercially from Perkin Elmer, Torrance, Calif., may be used.

In a preferred embodiment, a fluorescent dye and a quencher are attached to either side of the gamma-secretase substrate cleavage site of a gamma-secretase substrate. When the substrate is intact, the dye and quencher are in close proximity and no signal is produced from the assay. When the substrate is cleaved the quencher is removed from the dye, a signal results which can be monitored and quantified. This assay could be performed as an isolated biochemical assay *in vitro,* in cells and in

animal models *in vivo*. An alternate assay that could be used to monitor the efficacy of the drug in cells, animal models or human patients involves taking biopsies from diseased tissue and monitoring the cleavage of gamma-secretase substrates using conventional techniques such as detecting the presence and quantities of the substrates with antibodies.

Through research on malignant tumors, it has been found that certain tumors generate both angiogenesis-stimulating and inhibiting factors. This indicates that the angiogenic phenotype is the result of a balance between these positive and negative regulators of neovascularization. Novel means to increase angiogenesis may therefore be useful in conditions where increased angiogenesis is disfavored.

In light of the present inventive disclosure, numerous new methods can be developed. These methods can be based on the knowledge that gamma-secretase inhibitors, such as DAPT, initiate and increase angiogenesis. Certain of the new methods of the invention rely on comparisons of model systems' reactions to treatment with a gamma-secretase inhibitor relative to treatment with a control or no treatment. It is understood that practices commonly-used in the art are to be followed, for example, that except for the administration of gamma-secretase inhibitor the test conditions are otherwise as equivalent as possible. Certain other of the novel methods rely on evaluating one or more aspects or activities of gamma-secretase or the gamma-secretase activity in a model system. The skilled worker would determine which of these parameters to evaluate in order to best perform the novel methods.

Experimentation and analysis conducted during the pursuit of the present invention are described below as particular examples but not by way of limitation. Alternate methods known to skilled workers are within the scope of the invention. Unless otherwise noted, materials and equipment described herein are commercially available.

Example 1: Gamma-secretase inhibitor treated mice with oxygen induced retinopathy

Oxygen induced retinopathy mice
An animal model was used to determine the extent and effect of gamma-secretase inhibitors on the angiogenic process. Mice with oxygen induced retinopathy (OIR mice) are a commonly-used model and are described in the literature. They are at times referred to as retinopathy of prematurity models or ROP mice. The model takes advantage of the fact that full term mice pups are born with an immature retinal vascularization which matures during the first three weeks of postnatal growth.

Briefly, neonatal mice (NMRI/C57b1) from the same litter were placed, with their nursing mother, in a hyperoxic environment (75% oxygen) at age day seven. After exposure to the hyperoxic environment for 5 days, the 12-day old pups were removed to normal air. In the treated mice, DAPT was administered once daily during age days 12-16. Control pups were treated the same way but injected with the vehicle. At post

natal day 17 the mice were euthanized and the retinas were prepared for whole mount immunohistochemistry.

Preparation and Administration of DAPT
Except for the mode of injection, the stock solution of DAPT was prepared and administered essentially as described in Lanz *et al.,* (2003, J. Pharmacol. Exp. Ther. 305:864-71). In short, 5 mg DAPT was dissolved in 25μl 99.5% EtOH and then dissolved in 475μl Corn oil (Sigma, Catalog No. C8267). If precipitate formed, the solution was heated to 70°C for 2-3 minutes. The pups were injected subcutaneously once a day according to: V (μl) = weight (g) * 10, that is, 100 mg DAPT/kg body weight. Control pups were injected subcutaneously once a day with the same amount of vehicle (i.e. 5% EtOH in corn oil).

Whole mount immunohistochemistry
Eyes were fixed in 4% PFA in PBS at 4°C overnight and washed in PBS. Retinas were dissected, permeabilized in PBS, 1% BSA, and 0.5% TritonX-100 at 4°C overnight, rinsed in PBS, washed twice in PBlec (PBS, pH 6.8, 1% Triton-X100, 0.1 mM CaCl, 0.1 mM MgCl, 0.1 mM MnCl) and incubated in biotinylated isolectin B4 *(Bandeiraea simplicifolia;* L-2140; Sigma-Aldrich) 20g/ml in PBlec at 4°C overnight. After five washes in PBS, samples were incubated with streptavidin conjugates (Alexa 488, 568, or 633; Molecular Probes) diluted 1:100 in PBS, 0.5% BSA, and 0.25% Triton X-100 at 4°C for 6 hours. TO-PRO 3 (1:1,000; Molecular Probes) served for nuclear staining. After washing and a brief post fixation in PFA, the retinas were flat mounted using Mowiol/DABCO (Sigma-Aldrich).

As further described below, normal mice pups at 17 post natal days after oxygen induced retinopathy exhibit formation of avascular zones in the central, i.e., close to the optic nerve, areas of the retina. At the same time there is an increased vascular density in the peripheral parts of the retina. This can be quantified in the superficial capillary plexus by counting the number of capillary enclosed areas in the peripheral part of the retina.

Example 2: Evaluation of retinal vascularization and vessel density in gamma-secretase inhibitor treated OIR mice

Avascular retinal space
By comparing the retinas of control pups to those pups treated with gamma-secretase inhibitor, the effect of the inhibitor on angiogenesis was evaluated. As previously stated, otherwise untreated OIR mice pups will exhibit avascular zones in the central retina. Figure 1 shows the avascular space (avascular zones marked AZ in the figure) of a normal OIR mouse retina. In a surprising contrast, DAPT-treated OIR mice exhibited central retinal vascularization as shown in Figure 2. The treated mice almost completely lack the vascular-free zones. This is one example of the angiogenesis-initiating effect of gamma-secretase inhibitors.

Peripheral retina vascular density

Furthermore, at 17 post natal days after OIR, there is an increased vascular density in the peripheral parts of the retina. This can be quantified in the superficial capillary plexus by counting the number of capillary enclosed areas in the peripheral part of the retina. Figure 3 shows a control mouse retina, with asterisks pointing out capillary enclosed areas. The peripheral retina shown in Figure 4 is from a DAPT-treated mouse, showing a significant increase in capillary enclosed areas and therefore an angiogenesis-increasing effect. To further evaluate these results, the capillary enclosed areas were counted. The data presented in Figure 5 ($p<0.001$) reflects the near doubling of such areas in DAPT-treated mice.

Astrocyte interaction

Because retinal vessels grow in tight interaction with astrocytes, changes in the astrocytic network can lead to changes of the vasculature. To rule out the possibility that the observed increase in vessel density was due to an increased number of astrocytes, the retinas from control and DAPT-treated animals were stained with Glial Acidic Fibrillary Protein (GFAP) antibodies which specifically label astrocytes (DAKO). The GFAP antibodies were first diluted 1:75 and incubated at 4°C overnight, after washing the tissue was incubated with secondary antibody (anti-rabbit-Cy3red,Novakemi 111 165 144) diluted 1:100. Figures 6 and 7 show the astrocytic network of control and DAPT-treated mouse retinas, respectively. Little if any difference was observed in the density of GFAP positive astrocytes, therefore no vascular changes could be attributed to changes in the astrocytic network.

Example 3: Quantification of angiogenesis in gamma-secretase inhibitor treated OIR mice

Vascular tufts

Vascular tufts form in the retinas of OIR mice models. The tufts consist of endothelial cells growing in a small localized cluster above the inner limiting membrane and pouching into the vitreous. Figure 8 shows a retinal cross section with asterisks marking vascular tufts. Figure 9 shows a normal OIR mouse retina and numerous vascular tufts. Despite the increased angiogenic response seen in DAPT-treated OIR mice, a significant reduction in capillary tufts was observed as shown in Figure 10.

The numbers of capillary tufts can be quantified as a measurement of the pathological angiogenic response in the OIR model. This can be done, for example, with whole retinas stained with isolectin using a Nikon Microphot-FXA microscope and 4x magnification lens. Quantification of the results in this case are reflected in Figure 11 (p-value < 0.01).

Example 4: Evaluation of possible VEGF-A role in gamma-secretase inhibitor treated OIR mice

VEGF-A (Vascular Endothelial Growth Factor A) is an important factor for both physiological and pathological angiogenesis and has been shown to be important for the vascular changes seen in association with OIR. The increased vascularization in the DAPT-treated animals could potentially have been explained with an association to up-regulation of VEGF-A. To evaluate whether VEGF-A was a factor in the surprising angiogenesis initiation and increase observed with administration of DAPT, the amount of VEGF-A was quantified using an ELISA detecting VEGF-A protein (R&D Systems, Minneapolis, MN, USA). The protein was clearly detected in lysates of retina from both control and DAPT treated animals but no significant changes could be measured between the two groups. Supporting data are presented in Figure 12.

Example 5: Evaluation of retinal vessel density and architecture in gamma-secretase inhibitor treated non-OIR mice

To investigate if the increased retinal vascularization after DAPT treatment was restricted to the OIR, the effect of DAPT on physiological angiogenesis in new born mice was investigated. DAPT was administered to new born mice on postnatal days 3 and 4 and retinas were analyzed at day 5 as described above. As was observed with OIR mice treated with DAPT, there was an increased peripheral vascular density. This can be observed by comparing the degree of vascularization in the retina of control animals, Figures 13 and 14, with that of treated animals as shown in Figures 15 and 16. Except for the increased vascular density, the vascular architecture was normal. The growth of the vascular network towards the periphery and the arterio-venous specification were intact for DAPT-treated mice as with controls.

Example 6: Evaluation of gamma-secretase inhibitor in animal models of myocardial and ischemia, myocardial infarction, and peripheral ischemia

Mouse models of myocardial and limb ischemia
For therapeutic angiogenesis, a gamma-secretase inhibitor (e.g. DAPT) is delivered during the course of seven days to male Swiss mice aged 10-12 weeks. Thereafter, infarcted hearts are processed for morphometric analysis after immunostaining for endothelial thrombomodulin, which stains all vessels, or for smooth muscle x-actin, which stains mature SMC-covered vessels. See, for example, Lutgens E, *et al.*, Mar 1999 *Chronic myocardial infarction in the mouse: cardiac structural and functional changes*, Cardiovasc Res.;41(3):586-93.

To induce limb ischemia, unilateral right or bilateral ligations of the femoral artery and vein, proximal to the popliteal artery, and the cutaneous vessels branching from the caudal femoral artery side branch are performed without damaging the nervus femoralis. Gamma secretase inhibitors will be administered as described above. Two superficial preexisting collateral arterioles, connecting the femoral and sphenoid

artery, are used for analysis. Functional perfusion measurements of the collateral region can be performed using a Lisca PIM II camera (Gambro, Breda, the Netherlands) and analyzed as described (Couffinhal, T. *et al.* 1998 *Mouse model of angiogenesis.*, Am. J. Pathol. 152, 1667-1679). Perfusion, averaged from 3 images per mouse in the upper hind limb (adductor region where collaterals enlarge) or in total hind limb, is expressed as a ratio of right (ischemic) to left (normal) limb. Spontaneous mobility is scored by monitoring the gait abnormalities, the position of right foot in rest and after manipulation, and the "tail-abduction-reflex." Mice are scored 0 when one observation is abnormal and 1 when normal. Based on the results of the present invention, it is expected that such models of myocardial and limb ischemia will reveal DAPT-treated mice exhibit increased angiogenesis resulting in increased perfusion and formation of collateral vessels leading to increased healing/decreased tissue damage and increased function of the tissue.

Endurance exercise swim test for mice

Mice are conditioned for 9 days to swim in a 31°C controlled swimming pool under non-stressed conditions. At day 10, baseline exercise time for each mouse is determined using a counter-current swimming pool kept at 31°C; flow at 0.2 m/s. See, for example, Matsumoto, K., *et al.*, 1996 *An adjustable-current swimming pool for the evaluation of endurance capacity of mice.* J. Appl. Physiol. 81, 1843-1849. For determining maximal endurance exercise, i.e., the total swimming period until fatigue, the failure to rise to the surface of the water to breathe within 7 seconds is assessed. At day 11, the femoral artery is occluded as described above. At day 18 minipumps are removed under isoflurane anesthesia before endurance exercise.

Recovery of functionality is expressed as a ratio to the baseline exercise time. Fluorescent microspheres (yellow-green, 15 μm, 1 x 10^6 beads per ml, Molecular Probes, Eugene, Oregon) are administered after maximal vasodilation (sodium nitroprusside, 50 ng/ml, Sigma), processed, and flow was calculated (Carmeliet, P. *et al.* 1999, *Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188.* Nature Med. 5, 495-502). Bismuth gelatino-angiography is performed (see, for example, Carmeliet, P. *et al.*, 2001, *Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions.* Nature Med. 7, 575-583) and photo-angiographs (Nikon D1 digital camera) are analyzed in a blinded manner. Collateral side branches are categorized as follows: second-generation collateral arterioles directly branch off from the main collateral, whereas third-generation collateral arterioles are orientated perpendicularly to the second-generation branches. The number of collateral branches per cm length of the primary collateral arteriole is counted. Fluoroangiography is performed with a modified version of a described protocol (Carmeliet, P. *et al.*, 2001 *Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions.* Nature Med. 7, 575-583). Images are then reconstructed using, for example, a Zeiss LSM510 confocal laser microscope.

After perfusion-fixation, the 2 superficial collateral arterioles are post-fixed in paraformaldehyde 1% and paraffin-embedded. Twelve 5-μm cross-sections per superficial collateral, starting from the midzone and ranging over 1.95 mm to each end, are morphometrically analyzed. Collateral side branches are categorized as second generation (luminal area > 300 μm^2) or third generation (< 300 μm^2). Total perfusion area is calculated using the total sum of the side branch luminal areas. Capillary density is determined by immunostaining for thrombomodulin. Wall thickness of fully SMC-covered vessels is morphometrically measured on histological sections, after smooth muscle α-actin staining. For all treatment groups, six cross-sections (150 μm apart) are analyzed per main collateral. Only second-generation collateral arterioles larger than 300 μm^2 are included in the analysis. At least 10 measurements of wall thickness of the second-generation collateral arterioles are obtained. See, generally, Luttun A, *et al.*, Aug 2002, *Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1.*, Nat Med. 8(8):831-40; Lutgens E, *et al.*, Mar 1999, *Chronic myocardial infarction in the mouse: cardiac structural and functional changes*, Cardiovasc Res. 41(3):586-93. Based on the results of the present invention, it is expected that such endurance models will reveal DAPT-treated mice exhibit increased angiogenesis resulting in increased perfusion and formation of collateral vessels leading to increased healing/decreased tissue damage and increased function of the tissue.

The foregoing description and examples have been set forth merely to illustrate the invention and are not intended to be limiting. Since modifications of the disclosed embodiments incorporating the spirit and substance of the invention may occur to persons skilled in the art, the invention should be construed broadly to include all variations falling within the spirit and scope of the appended claims and equivalents thereof. The references disclosed herein, including U.S. Patents, are each specifically incorporated by reference in their entirety. However, the citation of such references shall not be construed as an admission that the references are prior art to the present invention.

Claims

1. An angiogenesis initiator or increaser, comprising a pharmaceutically-effective amount of a gamma-secretase inhibitor.

2. An angiogenesis initiator or increaser according to claim 1, wherein the gamma-secretase inhibitor is DAPT (N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-Butyl Ester).

3. An angiogenesis initiator or increaser according to either of Claims 1 or 2, further comprising a pharmaceutically acceptable carrier or adjuvant.

4. A method of influencing a disease state in a cell, a group of cells, or an organism, comprising:

administering at least one of a gamma-secretase inhibitor or a gamma-secretase pathway inhibitor to the cell, group of cells, or organism,

wherein the disease is selected from the group consisting of atherosclerosis, hemangioma, hemangioendothelioma, vascular malformations, warts, pyogenic granulomas, hair growth, Kaposi's sarcoma, scar keloids, allergic edema, neoplasms, psoriasis, decubitus or stasis ulcers, gastrointestinal ulcers, dysfunctional uterine bleeding, follicular cysts, ovarian hyperstimulation, endometriosis, neoplasms, preeclampsia, placental insufficiency, respiratory distress, ascites, peritoneal sclerosis, adhesion formation, metastatic spreading, coronary artery disease, ischemic heart disease, ischemic limb disease, obesity, rheumatoid arthritis, synovitis, bone destruction, cartilage destruction, osteomyelitis, pannus growth, osterphyte formation, cancer, aseptic necrosis, impaired fracture healing, hepatitis, pneumonia, glomerulonephritis, asthma, nasal polyps, liver regeneration, pulmonary hypertension, systemic hypertension, diabetes, retinopathy of prematurity, diabetic retinopathy, choroidal disorders, intraocular disorders (e.g. age related macular degeneration), leukomafacia, stroke, vascular dementia, disease, thyroiditis, thyroid enlargement, thyroid pseudocyst, tumor metastasis, lymphoproliferative disorders, lympgoedema, AIDS, and hematologic malignancies.

5. A method of increasing the angiogenic process in a cell, a group of cells, or an organism, comprising administering a pharmaceutical composition comprising a pharmaceutically effective amount of at least one gamma-secretase inhibitor or gamma-secretase pathway inhibitor to the cell, group of cells, or organism.

6. A method according to Claim 5, wherein the pharmaceutical composition is administered to prevent, treat, or cure a condition selected from the group consisting of atherosclerosis, hemangioma, hemangioendothelioma, vascular malformations, warts, pyogenic granulomas, hair growth, Kaposi's sarcoma, scar keloids, allergic edema, neoplasms, psoriasis, decubitus or stasis ulcers, gastrointestinal ulcers, dysfunctional uterine bleeding, follicular cysts, ovarian hyperstimulation, endometriosis, neoplasms, preeclampsia, placental insufficiency, respiratory distress, ascites, peritoneal sclerosis, adhesion formation, metastatic spreading, coronary artery

disease, ischemic heart disease, eschemic limb disease, obesity, rheumatoid arthritis, synovitis, bone destruction, cartilage destruction, osteomyelitis, pannus growth, osterphyte formation, cancer, aseptic necrosis, impaired fracture healing, hepatitis, pneumonia, glomerulonephritis, asthma, nasal polyps, liver regeneration, pulmonary hypertension, systemic hypertension, diabetes, retinopathy of prematurity, diabetic retinopathy, choroidal disorders, intraocular disorders (e.g. age related macular degeneration), leukomafacia, stroke, vascular dementia, disease, thyroiditis, thyroid enlargement, thyroid pseudocyst, tumor metastasis, lymphoproliferative disorders, lympgoedema, AIDS, and hematologic malignancies.

7. A method according to any of Claims 4-6, wherein the gamma-secretase inhibitor is DAPT or an analogue of DAPT.

8. A method for initiating or increasing angiogenesis in a cell, a group of cells, a tissue, or an organism, comprising inhibiting the gamma-secretase pathway in said cell, group of cells, tissue or organism.

9. A method for initiating or increasing angiogenesis in a cell, a group of cells, a tissue, or an organism, comprising inhibiting gamma-secretase in said cell, group of cells, tissue or organism.

10. A method according to either of Claims 8 or 9, wherein said method comprises at least one of reducing the level of expression of gamma-secretase in said cell, group of cells, tissue or organism, administering DAPT to said cell, group of cells, tissue or organism, administering an antibody against gamma-secretase to said cell, group of cells, tissue or organism, and delivering a vector to said mammal, said vector comprising a polynucleotide encoding at least one gamma-secretase inhibitor, operatively linked to a suitable promoter.

11. A method according to Claim 10, wherein said promoter is a tissue- or organ-specific promoter specific for a tissue or organ in which angiogenesis is to be initiated or increased.

12. A method for screening for a substance which initiates or increases angiogenesis, comprising:

measuring an activity of the gamma-secretase pathway in the presence of a candidate compound;

measuring an activity of the gamma-secretase pathway in the absence of a candidate compound; and

comparing said activity in the presence of a candidate compound with said activity in the absence of the candidate compound,

wherein a change in activity indicates that said candidate initiates or increases angiogenesis.

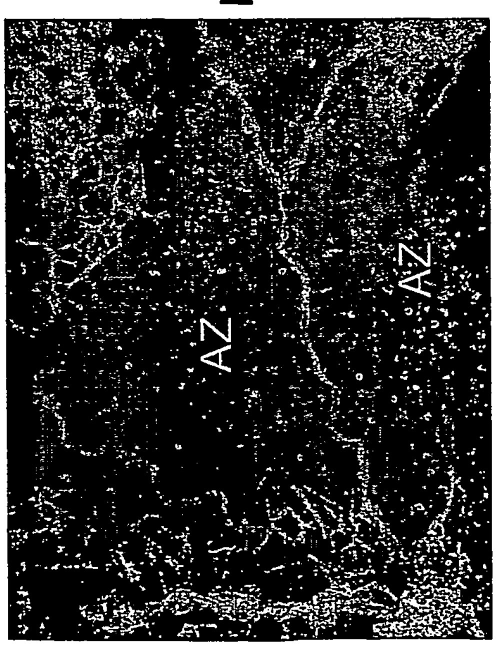

Fig 1

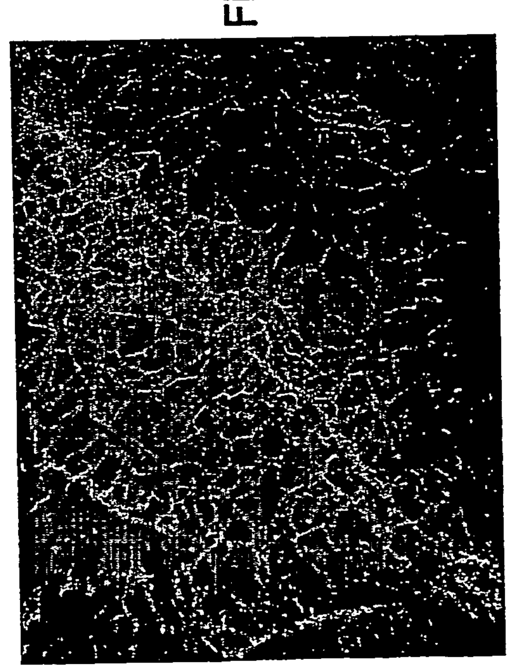

Fig 2

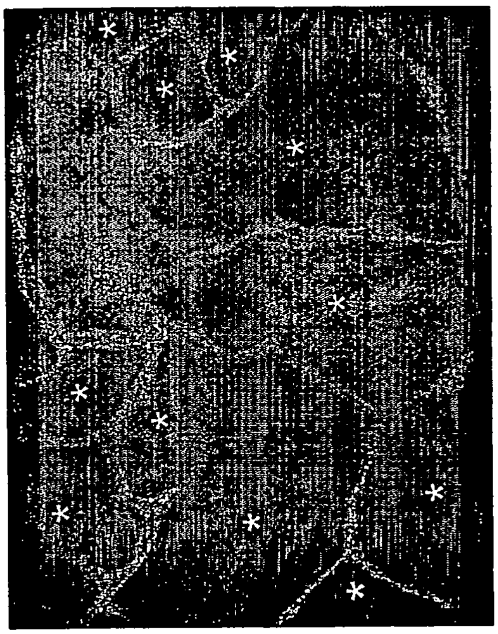

Fig 3

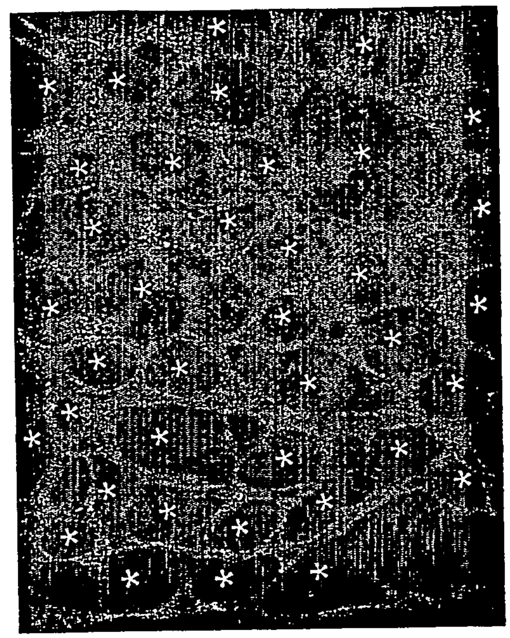

Fig 4

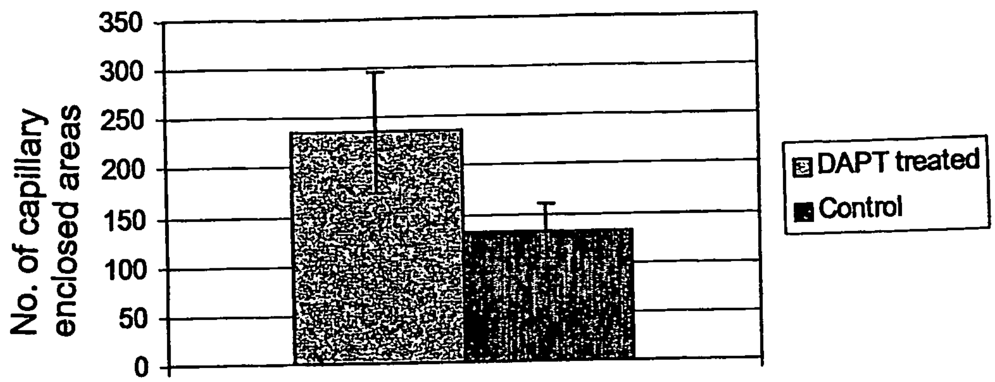

Fig 5

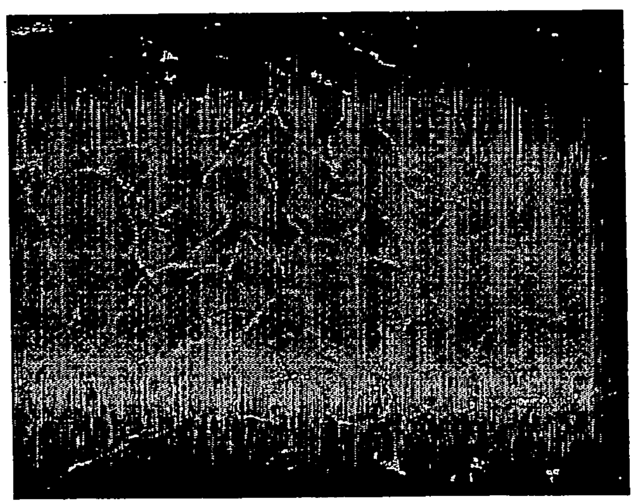

Fig 6

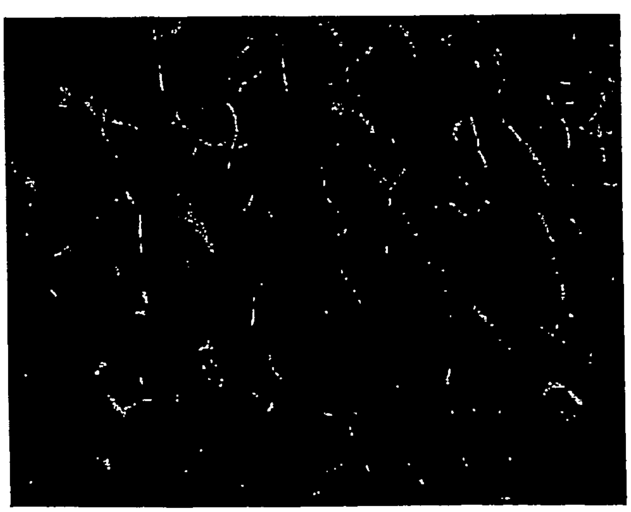

Fig 7

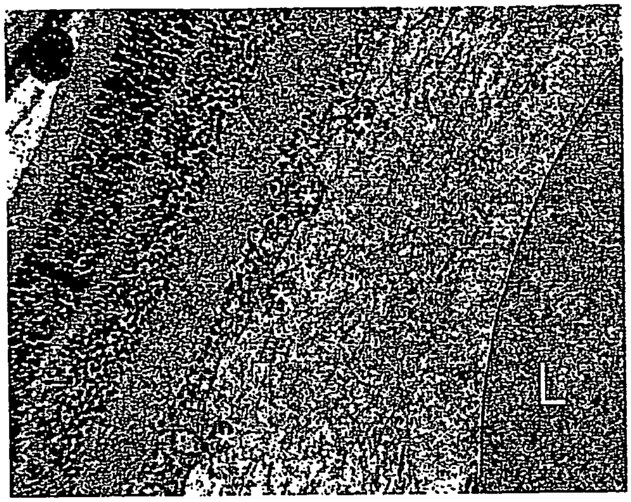

Fig 8

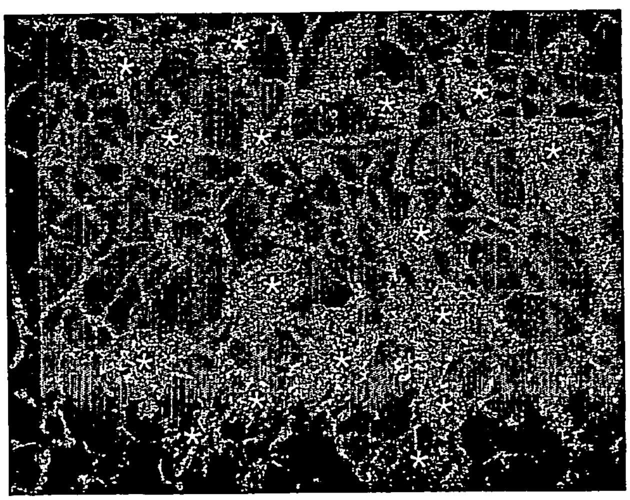

Fig 9

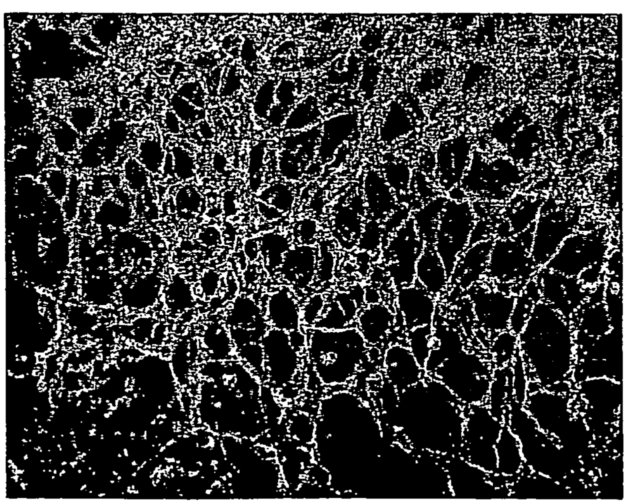

Fig 10

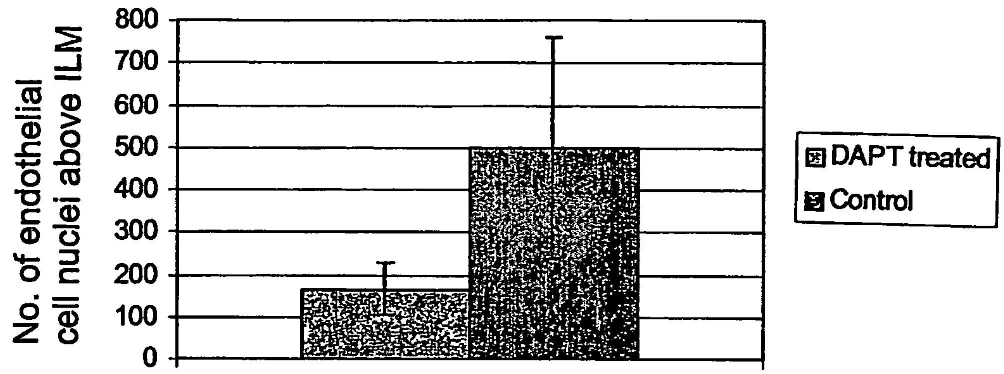
No. of endothelial cell nuclei above ILM
800
700
600
500
400
300
200
100
0
DAPT treated
Control

Fig 11

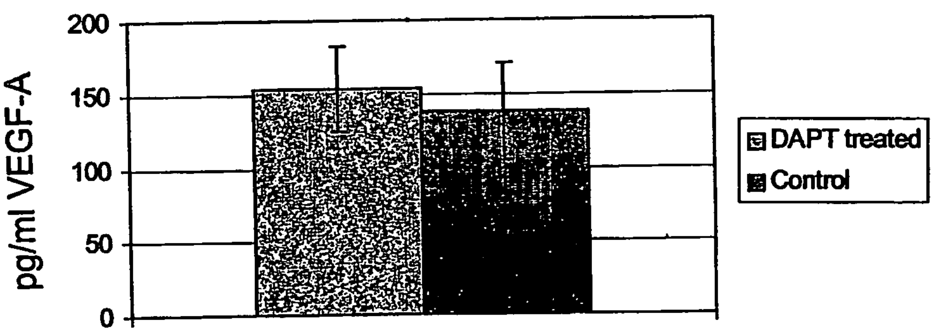
pg/ml VEGF-A
200
150
100
50
0
DAPT treated
Control

Fig 12

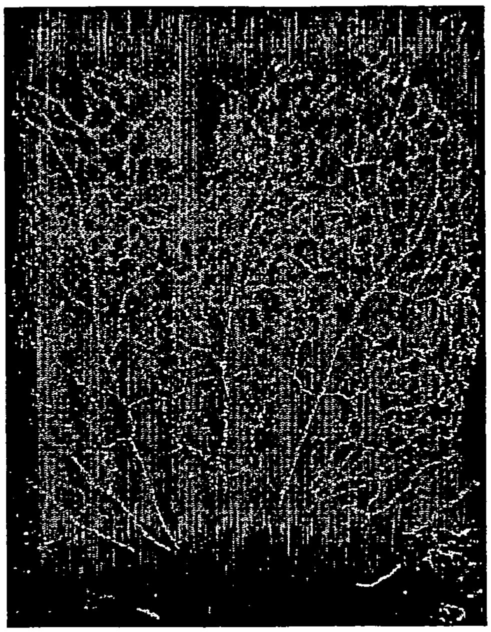

Fig 15

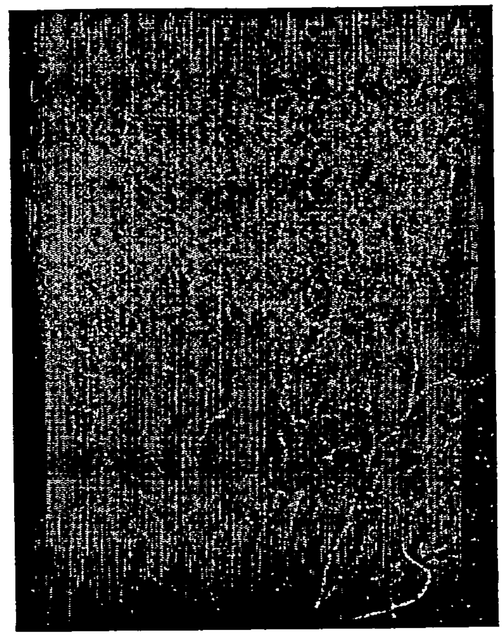

Fig 16

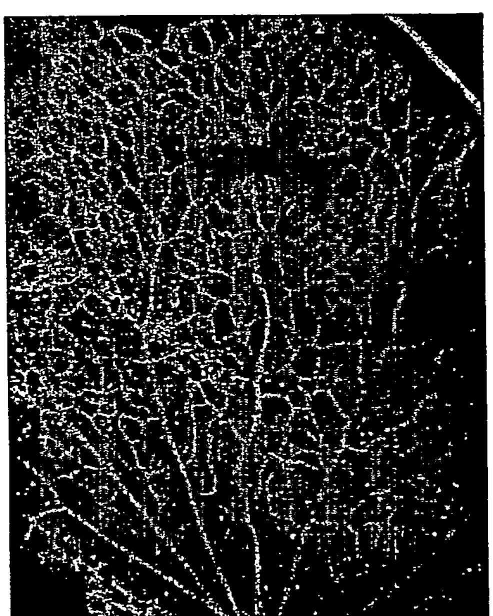

Fig 13

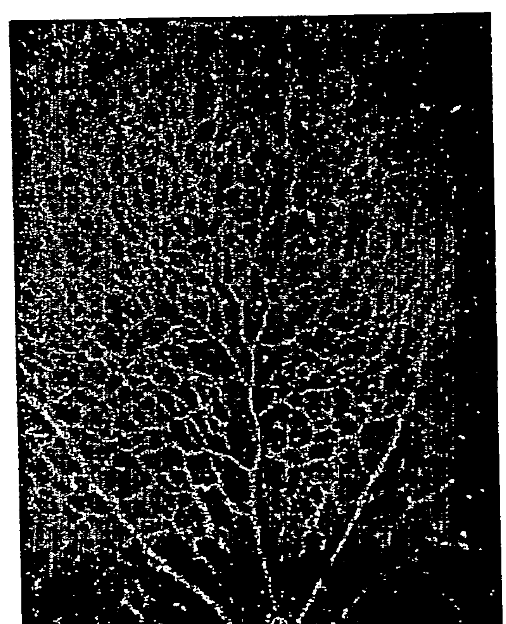

Fig 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 2004/001146

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: G01N 33/68, A61K 38/00, A61K 39/00, A61P 9/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: G01N, A61K, A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Mitsunari Nakajimaa, Shigeki Yuasa , Masaya Uenoc, Nobuyuki Takakura. Haruhiko Kosekiu, Takuji Shirasawa, "Abnormal blood vessel development in mice lacking presenilin-1", Mechanisms of Development 120 (2003): 657-667, see the whole document --	1-12
Y	David M. A. Mann, Stuart M. Pickering-Brown, Ayano Takeuchi, Takeshi Iwatsubo, "Amyloid Angiopathy and Variability in Amyloid Beta Deposition Is Determined by Mutation Position in Presenilin-1-Linked Alzheimer's Disease", American Journal of Pathology, Vol. 158, No. 6, June 2001: 2165-2175, see page 2165 --	1-12

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 November 2004	0 4 -11- 2004
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer MALIN SÖDERMAN/BS Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 2004/001146

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Craig A. Micchelli, William P. Esler, W. Taylor Kimberly, Christine Jack, Oksana Berezovska, Anna Kornilova, Bradley T. Hyman, Norbet Perrimon, Michael S. Wolfe", "Gamma-Secretase/presenilin inhibitors for Alzheimer's disease phenocopy Notch mutations in Drosophila", The FASEB Journal, Vol. 17. January 2003: 79-81, see page 79	1-3
Y		4-12
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A	Dong-Gyu Jo, Jae-Woong Chang, Hyun-Seok Hong, Inhee Mook-Jung, Yong-Keun Jung, "Contribution of presenilin/gamma-secretase to calsenilin-mediated apoptosis", Biochemical and Biophysical Research Communications 305 (2003): 62-66, see the whole document	1-12
	--	
A	Anthony H Vagnucci, William W Li, "Alzheimer's disease and angiogenesis", The Lancet, Vol. 361, February 2003: 605-608, see the whole document	1-12
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X	WO 02018544 A2 (LOYOLA UNIVERSITY CHICAGO), 7 March 2002 (07.03.2002), claims 30-33	4-6
A		1-3,7-12
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X	BIOSIS, accession no. PREV200300356701, Jundth Franziska, Arnold Wolfgang, Mathas Stephan, Wolfe Michael, Foster Reinhold, Dorken Bernd, "Novel gamma-Secretase inhibitor DAPT Blocks Activated Notch Signaling and Controls Tumor Cell Growth in Hodgkin and Anaplastic Large Cell Lymphoma, Blood, November 16, 2002, Vol. 100, No 11, PG-Abstracts No. 594, see abstract	4-7
A		1-3,8-12
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 2004/001146

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 4-12
   because they relate to subject matter not required to be searched by this Authority, namely:
   see extra sheet

2. ☒ Claims Nos.: 1-3
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
   see extra sheet

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Box II.1

Claims 4-12 relate to methods of treatment of the human or animal body by surgery or by therapy or diagnostic methods practiced on the human or animal body (PCT Rule 39.1(iv)). Nevertheless, a search has been executed for these claims. The search has been based on the alleged effects of the compounds or compositions. It is not clear from claims 8, 9, 12 that the methods are performed in vitro.

Box II.2

Present claims 1-3 relate to a composition defined by reference to a desirable characteristic or property, namely "an angiogenesis initiator or increaser" and "a gamma-secretase inhibitor". The claims cover all compositions having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and / or disclosure within the meaning of Article 5 PCT for only a very limited number of such compositions. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compositions by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible.

Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the compositions comprising gamma-secretase DAPT, described in claim 2. Further, the search has covered the general aspects of the invention to some extent, although it lacks the necessary precision in the definition of the subject matter. Consequently, the search for the general concept of "angiogenesis initiator or increaser" and "a gamma-secretase inhibitor" will retrieve a pertinent document only if this concept is described in general terms in a reference. Specific solutions previously known and falling under the general concept - but failing to mention this fact - are likely not to be revealed in such a search.

WO	02018544	A2	07/03/2002	NONE